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MicroRNA-210 enhances the therapeutic potential of bone marrow-derived circulating proangiogenic cells in the setting of limb ischemia.

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Abstract

Therapies based on circulating proangiogenic cells (PACs) have shown promise in ischemic disease models, but require further optimization to reach the bedside. Ischemia-associated hypoxia robustly increases microRNA-210 (miR-210) expression in several cell types, including endothelial cells (ECs). In ECs, miR-210 represses EphrinA3 (EFNA3), inducing proangiogenic responses.

This study provides new mechanistic evidences for a role of miR-210 in PACs. PACs were obtained from either adult peripheral blood or cord blood. miR-210 expression was modulated with either an inhibitory complementary oligonucleotide (anti-miR-210) or a miRNA mimic (pre-miR-210). Scramble and absence of transfection served as controls. As expected, hypoxia increased miR-210 in PACs. *In vivo*, migration toward and adhesion to the ischemic endothelium facilitate the pro-angiogenic actions of transplanted PACs. *In vitro*, PAC migration toward SDF-1α/CXCL12 was impaired by anti-miR-210 and enhanced by pre-miR-210. Moreover, pre-miR-210 increased PAC adhesion to ECs and supported angiogenic responses in co-cultured ECs. These responses were not associated with changes in extracellular miR-210 and were abrogated by lentivirus-mediated EFNA3 overexpression. Finally, *ex-vivo* pre-miR-210 transfection predisposed PACs to induce post-ischemic therapeutic neovascularization and blood flow recovery in an immunodeficient mouse limb ischemia model.

In conclusion, miR-210 modulates PAC functions and improves their therapeutic potential in limb ischemia.

Keywords: microRNA-210; EFNA3; bone marrow-derived circulating cells; angiogenesis; limb ischemia; cell therapy.

Introduction

It has been estimated that more than 200 million people worldwide are affected by peripheral arterial disease (PAD).[1] Critical limb ischemia (CLI), the most serious form of PAD in the legs, requires foot amputation in 25% of subjects within one year from the diagnosis, and 25% of the patients with CLI will die during the same period.[2] Revascularization unsuitability or failure results in more severe outcomes in terms of major amputation and mortality rates, especially in subjects with diabetes mellitu.[3] Even if successful, revascularization does not prevent systemic complications (infarction or stroke).[4] Regenerative medicine could offer new solutions to this outstanding clinical need. Restoring tissue homeostasis after an ischemic event requires the control of vascular and muscular protection and regeneration, involving complex multicellular processes brought about by cells resident in muscles [vascular endothelial cells (ECs), pericytes and satellite cells] and bone marrow (BM)-derived proangiogenic circulating cells].[5-7] These concepts are at the basis of the development of cell-based therapeutic angiogenesis for patients suffering of PAD and CLI. The outcome of a recent meta-analysis revising the efficacy of cell injection in no-option CLI patients has showed that cell therapy enhances wound healing and reduces amputation probability by 59% and 37%, respectively.[8] The therapeutic value of blood circulating BM-derived cells may be dependent on the abundance and quality of proangiogenic cells (PAC). This concept was raised by the discovery about 2 decades ago of the postnatal vasculogenic and angiogenic potential of a subpopulations of circulating mononuclear cells (MNCs) of BM origin that express CD34 and the VEGF-A receptor KDR.[9] One approach to study these PACs, also previously known as Endothelial Progenitor Cells (EPCs), is to enrich them by culture using a medium containing EC growth factors. PACs are positive for CD45, CD34, CD31 and KDR and uptake acetylated low density lipoproteins (LDL). When injected in immunocompromised animal models of limb or myocardial ischemia, PACs have been reported to physically adhere to the endothelium and integrate in blood vessels; albeit their ability to differentiate in mature ECs is now considered limited. Additionally, PACs support angiogenesis *via* paracrine actions.[10] PACs represent an easy accessible, ethically justifiable and valuable study model for autologous cell

therapy strategies.[11, 12] However, the translational potential of autologous adult PACs is tempered by their low abundance in the circulation, difficulties to expanding the cells *in vitro* and the negative impact of associated risk factors on the cell regenerative potential. In this context, in 2012, we proposed the possibility to improve the phenotype of adult PAC before transplantation by using microRNAs (miRs) that control intrinsic stem cell function and angiogenesis,[13] but we did not investigate miR-210.[14, 15] Umbilical cord blood (UBC) represents an alternative source of PAC. Indeed, PACs are more abundant in the cord blood than in the adult blood and UBC-PACs can be more easily expanded in culture, offering obvious advantages for performing *in vivo* transplantation studies.[16, 17] Additionally, UCB-PACs can be banked for future therapeutic applications benefitting the donors. UCB-PACs banking can be particularly indicated in subjects born with known congenital defects in their heart and vasculature. These patients will enter a journey of investigations and often repeated surgeries and interventions and have a higher risk of developing ischemic disease.[18]

Hypoxia, *via* HIF-1α, robustly increases intracellular miR-210 expression in several tissues and cells, including ECs.[14, 15] In ECs, miR-210 activates survival pathways[19-22] and enhances migratory and proangiogenic capacities.[21, 23-30] We previously showed that miR-210 modulates EC by inhibiting Ephrin-A3 (EFNA3), a glycosylphosphatidyl-inositol-linked membrane-bound ligand.[21] Ephrins are a family of cell surface ligands that interact with Eph tyrosine kinases receptors to mediate intercellular adhesion and repulsion. Ephrin-Eph interactions guide migration and positioning of the cells for proper tissue patterning during vascular development and postnatal angiogenesis. Interestingly, even if EFNA3 is inhibited by miR-210, EFNA3 was found expressed at very high levels in ischemic tissues.[31] In a cancer setting, it was recently shown that hypoxia regulates EFNA3 expression through a non-direct mechanism that involves the HIF-mediated transcriptional induction of a novel family of long-noncoding RNAs (lncRNAs) from the *EFNA3* locus.[32] In turn, lncEFNA3 would favor EFNA3 protein accumulation acting at the post-transcriptional level. [32]

This study has mechanistically investigated the expression and function of miR-210 in cultured PACs and trialled the therapeutic potential of miR-210 overexpressing PACs in an immunocompromised mouse model of limb ischemia.

Results

Hypoxia increases miR-210 in human PACs

Adult PB-PACs were obtained from mononuclear cells (MNCs) of healthy donors by culture selection in the presence of EC growth factors-rich medium. Similar to our previous studies, [13, 33] cultured PB-PACs were positive for CD45, CD34, Kinase Insert Domain Receptor (KDR), and CD14 (LPS receptor, expressed prevalently on monocytic cells) (**Figure 1A**). Following hypoxia, PB-PACs increased both the primary transcript (pri-miR-210) and mature forms of miR-210 (**Figure 1B i and ii**). We tested whether hypoxia increased the secretion of mature miR-210 in the conditioned medium, but no significant differences were observed in either the unfractionated medium or in the extracellular vesicles (EVs) (**Figures 1B iii and iv**), suggesting some level of miR-210 retention in the parental cells.

Modulation of miR-210 levels by pre- and anti-miR-210

To study whether miR-210 modulation had an impact on human PAC biofunctions, we transfected PB-PACs with a synthetic miR-210 sequence (pre-miR-210) or chemically modified complementary oligonucleotides (anti-miR-210) to enhance or inhibit its levels, respectively. As shown in **Supplementary figure I**, both interventions efficiently modulated miR-210 levels of PB-PACs cultured.

miR-210 does not modulate the expression of HIF-1α–regulated genes in PACs

The hypoxia responsive factor HIF-1α modulates miR-210 directly [34] and it was proposed that miR-210 regulates a feed-forward mechanism involving HIF-1α -regulated genes.[35-37] However, in PACs, the expression of genes under the control of HIF-1α such as Glucose transporter 1 (*GLUT-1*), Aldolase, Fructose-Bisphosphate C (*ALDO-C*), *VEGF-A*, and Adrenomedullin (*ADM*) were not affected by forced miR-210 expression changes (**Supplementary figure II**), which suggests the absence of loop in which miR-210 potentiates HIF-1α activity. The data discourages the

speculation that such genes could mediate the responses induced by PAC expressing miR-210 in the adopted experimental conditions.

SDF-1α-directed migration of PB-PACs is sensitive to miR-210 levels

After an ischemic insult, chemotactic factors are released in the circulation and PB-PACs follow the chemokine gradient to migrating toward and home at the injured tissue.[38] SDF-1α (CXCL12) is considered to be the prototypical pro-migratory chemokine.[39] As shown in **Figure 2A**, we first validated that cultured PB-PACs migrated towards SDF-1α both in normoxic and hypoxic conditions. Moreover, pre-miR-210-transfected PB-PACs increased SDF-1α-directed cell migration both in normoxia (p=0.0032 vs. vehicle and p=0.0058 vs. SDF-1α-SCR) and in hypoxia (p=0.0004 vs. vehicle and p=0.0013 vs. SDF-1α-SCR). Consistently, blocking miR-210 with anti-miR reduced PB-PAC migratory ability in the presence of the chemoattractant both under normoxia and hypoxia (p=0.0105 and p=0.0250 vs. SCR, respectively). The migration toward SDF-1α requires the activation of SDF-1α cognate receptor C-X-C chemokine receptor type 4 (CXCR-4) in the parent cells.[40] Worth of note, hypoxia reportedly increases CXCR4 expression in progenitor cells.[41] Therefore, we verified if CXCR4 expression was changed in miR-210-transfected-PB-PACs. CXCR4 was effectively induced by hypoxia in PB-PACs, but not further regulated by the miR-210 changes induced by transfection with pre- or anti-miR-210 (**Supplementary figure III A&B**).

miR-210 modulation affects the interaction of PB-PACs and endothelial cells in co-culture

We observed morphological changes in adult PACs overexpressing miR-210, as highlighted by the staining for the cytoskeletal protein phalloidin (**Figure 2B**), with an increased number of flat and round-shaped cells both in normoxic or hypoxic conditions (p=0.0176 and p=0.0358 vs. SCR, respectively). In addition, in miR-210-transfected PB-PACs hypoxia induced a further increase in the number of round-shaped cells (p=0.0493 vs. normoxia). These evidences suggest a potential effect of miR-210 on cell adhesion. Indeed, to support ECs in the angiogenic process, PB-PACs need first to adhere to the endothelium. Thus, we measured the number of PKH26 red-stained PB-

PACs that were efficiently attached to a monolayer of human umbilical vein EC (HUVECs) after one hour of co-culture. Hypoxia promoted PB-PAC adhesion to HUVECs (p=0.0311 vs. normoxia). Of note, miR-210 overexpression alone (p=0.0052 vs. SCR) or in combination with hypoxia (p=0.0313 vs. SCR) increased PACs adhesion. In addition, inhibiting miR-210 in PB-PACs with anti-miR prevented the enhancement of hypoxia-induced PB-PACs adhesion to ECs (**Figure 3A**).

Next, we investigated the possibility that PB-PACs transfected with pre-miR-210 could better support HUVEC capacity to form network structures on a Matrigel layer, a model of *in vitro* angiogenesis. When HUVECs were cultured with miR-210-overexpressing PACs, they showed a higher networking capacity both under normoxia and hypoxia (p=0.0241 and p=0.0003 vs. SCR-PACs, respectively) (**Figure 3B**). Green PKH67-labeled- miR-210-transfected-PACs locate all around red PKH26-labeled-HUVECs confirming their cooperation in HUVECs networking on Matrigel suggesting that cell-cell contact is necessary for miR-210-dependent stimulation of angiogenesis by PB-PACs. Supporting this hypothesis, neither the complete cell conditioned medium (CCM) of miR-210-transfected-PB-PACs or isolated EVs obtained from miR-210-tranfected-PB-PACs increased EC networking (**Supplementary figure IV A&B**). Accordingly, the number of exosome-sized EVs measured by Nanosight technology was similar in CCM from SCR, pre-miR-210 and anti-miR-210 transfected PB-PACs (**Supplementary figure IV C**).

EFNA3 overexpression prevents miR-210-induced migration and adhesion of PACs *in vitro*

EFNA3 is directly targeted by miR-210 for repression.[21, 42, 43] In ECs, miR-210 does not reduce mRNA level, but decreases EFNA3 protein.[21] In line with that, both EFNA3 mRNA and miR-210 were found upregulated in the ischemic brain.[31] Interestingly, in hypoxic metastatic tumors, EFNA3 protein expression was reported to be additionally regulated by HIF-induced expression of lncRNAs transcribed from the *EFNA3* locus.[32] Here, we investigated whether a similar mechanism occurred in PACs. Using qPCR primers able to discriminate the mRNA and lncRNA transcripts (**Supplementary figure VA**), we observed that in comparison to EFNA mRNA, lncEFNA3 expression was negligible in PACs (data not shown). Moreover, in PACs neither hypoxia nor forced

miR-210 expressional changes affected the expression of EFNA3 mRNA or of lncEFNA3 (**Figure 4A i and ii**). However, EFNA3 protein levels, measured by immunofluorescence staining, were responsive to miR-210 expression (**Figure 4B**). To study whether EFNA3 repression in PB-PACs is important for miR-210-induced migration and adhesion, we attempted to rescue EFNA3 expression using a lentiviral vector (LV). The successful increase in EFNA3 mRNA after LV-mediated gene transfer of PACs was confirmed by qPCR (**Figure 5A&B**).

Figure 5C shows that the concomitant up-regulation of the duo EFNA3/miR-210 prevented the pro-migratory function of miR-210, especially in the presence of the chemoattractant SDF-1α. A similar effect was also observed in the cell adhesion protocol (**Figure 5D**), where pre-miR-210 transfection increases PB-PACs adhesion. Interestingly, EFNA3 overexpression prevented miR-210-induced adhesion to HUVECs (Figure 5D). EFNA3 action can be mediated by the engagement of its EPHA4 receptor.[44] Therefore, we additionally analyzed the expression of EPHA4 in PACs. We could not detect any significant EPHA4 expressional change associated with PAC transfection of SCR, pre-miR-210 or anti-miR-210 under normoxia and hypoxia (**Supplementary figure VB**). Collectively, the above data indicate that miR-210 upregulation targets EFNA3 and this decrease is necessary for miR-210 pro-migratory and pro-adhesive functions.

miR-210 enhances the therapeutic potential of PACs in a mouse model of limb ischemia

For the *in vivo* protocol, we employed UCB-PACs. The UBC-PACs were first characterized for the expression of the classical markers already reported in PB-PACs (see **Figure 1A**). Consistently with the literature, a high proportion of UCB-derived cells (40%) were CD34 positive and (40%) of the cells were KDR positive, a lower proportion of the cells were CD45, while only few cells expressed CD14 (**Supplementary figure VI A**).[17, 45] Next, we sought confirmation that the new model of UCB-PACs behaved similarly to PB-PACs characterized in this study. In details, UBC-PACs responded to hypoxia with increased levels of miR-210 (**Supplementary figure VI B**). Furthermore, UCB-PACs could be efficiently transfected with pre-miR-210 or anti-miR-210, overexpressing or inhibiting miR-210, respectively (**Supplementary figure VI C**). Functionally, we

also verified that in UCB-PACs pre-miR-210 transfection increased the capacity to support HUVEC networking on Matrigel, while anti-miR-210 elicited the opposite effect (**Supplementary figure VI D**).

Next, the therapeutic potential of UCB-PACs expressing different miR-210 levels was assessed *in vivo*. Transplantation of UCB-PACs over-expressing miR-210 into ischemic muscles improved post-ischemic blood flow recovery (**Figure 6A**) and increased microvascular density in ischemic muscles (**Figure 6B**). In this experimental setting, we did not observe therapeutic responses associated with injection of PACs transfected with either scrambled or anti-miR-210 oligonucleotides (**Figure 6B**). Comparisons between responses induced by naïve UCB-PACs and EBM-2 control medium showed no difference, suggesting that the lack of therapeutic effect of PACs transplantation alone was not due to a negative effect of the transfection on the cell function (**Supplementary figure VII A and B**). Even if we cannot provide data on circulating miR-210 in immunocompromised mice receiving PAC transplantation, we measured miR-210 by qPCR in the plasma of in C57BL/6 mice subjected for 3 days to experimental ischemia (**Supplementary figure VIIC**). Data confirm an increase of miR-210 also in the plasma of ischemic mice.

Discussion

In this investigation, we demonstrated the potential of miR-210 to improve migratory and adhesive abilities of PACs, with positive consequences on angiogenesis *in vitro* and *in vivo*.

miR-210 is historically regarded as the master hypoxamiR. Indeed, endogenous miR-210 has been proven to be dramatically increased by hypoxia in virtually any tested cell type.[14, 15] Adult and cord blood PACs are not exception, as demonstrated here for the first time. Since high miR-210 levels in ECs were associated with activation of angiogenesis, we investigated whether bearing high miR-210 was stimulating also PAC functions, with the final goal of improving cell-based regenerative therapies approaches.[46] Specifically, *in vitro*, we worked with PACs derived from two sources: adult PB and UCB. By contrast, for *in vivo* experiments, we focused on UCB-PACs, capitalizing from the University of Bristol research program employing perinatal material for stem cells and tissue engineering therapies dedicated to patients born with congenital heart defects, although, the cells used here were all from healthy donors. We previously characterized human adult PACs in several studies and demonstrated that these cells express a series of miRs that regulate angiogenesis. We already proved that PACs can be engineered with either anti-miRs (to miR-15a and 16) or tissue kallikrein to improve their migratory capacity and *in vivo* proangiogenic potential.[13, 33] Here, we provide novel evidence that miR-210 overexpression enhanced PAC migration *in vitro*. This is associated with a remodeling of the cell shape and reduced EFNA3 protein expression. EFNA3 is a well characterized miR-210 target.[21, 31, 42, 43] Gomez-Maldonado *et al* recently proposed that lncEFNA3 isoforms are induced by hypoxia and positively regulate EFNA3 expression.[32] However, in PACs, lncEFNA3 expression was much lower than the EFNA3 mRNA and lncEFNA3 expression was not modulated by hypoxia or miR-210.

Overriding the miR-210-induced EFNA3 down-modulation was shown to prevent EC chemotaxis/angiogenesis.[21, 42] We confirmed this in PACs by simultaneously overexpressing miR-210 and EFNA3. In details, we showed that LV-induced increase in EFNA3 were sufficient to contrast the pro-migratory and pro-adhesive effects induced by premiR-210 transfection in PACs. The data provide evidence of the importance of EFNA3-miR-210 interaction in regulating the

behavior of miR-210-engineered PACs. However, further experiments are needed to clarify the EFNA3-miR-210 relationship under physiological conditions.

The possibility to use miR engineering to manipulate stem cells into more powerful therapeutic tools is attractive. We had already validated the feasibility and potential of manipulating miR-17 expression in UCB-PACs.[47] Moreover, miR-210 overexpression already proved utilitarian in mesenchymal stromal cells (MSCs).[22, 48] miR-210 modulation was associated with increased cell survival, in keeping with the inhibition of the miR-210 target, caspase-8-associated protein-2.[35] In PACs, miR-210 expressional changes did not impact cell proliferation and survival (data not shown), suggesting that miR-210 function is highly context-dependent.[14, 49]

PACs have been shown to activate resident ECs, at least in part through vesicle-mediated transfer of miRs.[50, 51, 52] Moreover, increased miR-210 microvesicle-associated levels have been observed in a variety of cell types exposed to hypoxia.[42, 53-56] However, at difference with recent EV literature, [57-60] the EV cargo of PACs did not appear to reflect miR-210 changes in the parent cells. Moreover, our data provide evidence against the hypothesis that PAC-released extracellular miR-210 of either endogenous or exogenous origin mediates the angiogenic responses to PACs. This represent a relevant control, because it allows us to exclude that miR-210 released by overloaded PACs after premiR-210 transfection could account for the response observed in ECs co-cultured with PACs.

miR-210 overexpression in PB-PACs stimulated changes in their cytoskeleton and promoted angiogenesis response by co-cultured EC. However, anti-miR-210 treatment of PACs did not impact on this effect. While we do not have an exhaustive explanation of the underlying mechanisms, we speculate that the level of miR-210 necessary to promote such PAC actions are maintained after anti-miR-210. Another possible interpretation is that miR-210 might be sufficient, but not necessary for certain PB-PAC functions. It is also worth noting that the response to anti-miR-210 might be cell-type dependent. In fact, miR-210 inhibition decreased the ability of UBC-PACs to stimulate the capillary-like tube formation in co-cultured ECs.

To validate *in vivo* the proangiogenic function played by miR-210 in PACs, we employed UCB-PACs, which are currently considered a particularly attractive cell system for clinical application.[16,

[17] Additionally, it was reported that, in comparison with adult PACs, human UBC-PACs induce growth of more durable blood vessels in mouse (up to 4 months vs. 3 weeks for adult cells PACs).[61] Moreover, data obtained studying UBC-PACs might be more easily transferred to a future clinical setting, since the UCB is currently an approved source of cell for regenerative therapy and it is banked in several countries.[18] *In vitro* experiments confirmed that in UBC-PACs miR-210 was readily induced by hypoxia and that its expression stimulated the network formation by co-cultured ECs. Since we found that the miR-210-PACs supports angiogenesis by cell to cell contact *in vitro*, we injected the cells directly into the ischemic tissue, to allow cell contact to happen in the ischemic limb vasculature. We acknowledge that this site of injection creates an unfavorable environment for cell survival, as previously described.[62] Pre-miR-210-overexpressing UBC-PACs increased post-ischemic reparative angiogenesis and blood flow recovery. Interestingly, the effect seems to be stronger on small arteriole development as compare to capillary density. We believe this reveals an interesting effect of miR-210-engineered PACs for which we do not have a clear mechanism, and that would be interesting to explore further.

We could not measure a change of miR-210 expression *in vitro* in the conditioned medium or in the extracellular vesicles of miR-210-PACs. Nonetheless, we cannot exclude that after transplantation, PAC start to release miR-210, contributing to the effect on vascular repair. In line with this possibility, we found that endogenous cells, of not yet defined types, appear to contribute to limb ischemia-induced increases in circulating miR-210 levels observed in mouse PB-derived plasma. Naïve PB-PACs and CB-PACs have shown beneficial effects in preclinical and clinical studies.[63] We have been previously unable to replicate such responses with adult PB-PACs.[13] Similarly, we here report absence of *in vivo* therapeutic responses to untreated UCB-PACs. This could be explained taking into account that different studies might differ for methods of cells isolation, surgical techniques to induce limb ischemia, animal background and sex, site of cell injection. Moreover, the number of cells injected in mice usually varies between $2x10^5$ to $1x10^6$ cells.[47, 64, 65] We injected $2x10^5$ cells, which is at the lower boundary of the aforementioned range.

Altogether, our data point to miR-210 as way to improve PAC functional activities. We therefore propose that miR-210-engineering of PACs should be further explored as a way to improve cellular regenerative therapies in ischemic diseases. miR-210 overexpression in PACs and other regenerative cells could also aid in tissue engineering. Example scenarios of the latter would be using miR-210-expressing PACs to support vascularization of scaffolds, as well as of vascular and heart valve conducts, thus responding to the demands regenerative medicine must meet to correct acquired defects (like CLI and ischemic heart disease), while expanding to address the needs presented by surgery for congenital heart and vessels defects.

Materials and Methods

Cell isolation, culture, and treatments

Human blood circulating PACs were isolated and cultured as previously described.[13, 33] form a small cohort of healthy subjects. The study protocol complied with principles of the Declaration of Helsinki, was covered by institutional ethical approval (11/2015 Cardiovascolare). For this study 25 patients were enrolled with a mean age (SEM) of 41.5 (2.5) years. The 40% of the patients were male and the 60% were female. The collection of human umbilical cord blood used in this study was approved by the North Somerset and South Bristol Research Ethics Committee (Research Ethic Committee –REC- reference 11/H0107/4). A total of 11 donors were recruited at a gestational age comprised between 39 and 40 weeks. The cells were isolated following the same protocol than used for the isolation of PACs from adult peripheral blood. Briefly, human umbilical cord blood mononuclear cells were isolated by gradient centrifugation on Histopaque-1077 density medium (Sigma). For enrichment of the PACs, $1x10^7$ mononuclear cells/well were plated on fibronectin (Sigma)-coated 6-well plates (BD Falcon) and cultured for 4 days in EBM-2, supplemented with EGM-2 MV SingleQuots, 10% FBS (EGM-2MV; Cambrex) and 1% penicillin/streptomycin. At that time, non-adherent cells were removed by 2 washes of PBS. Cells were then cultured for 6-8 days before being transfected with a non-targeting sequence, also identified as scramble, SCR, throughout the manuscript (AM4611, Silencer® Negative Control, Ambion), pre-miR-210-3p (PM10516, Ambion) or anti-miR-210-3p (AM10516, Ambion) 50nmol/L using Gensilencer Transfection reagent (Genlantis) using the manufacturer's protocol. Three days after transfection, cells were submitted to hypoxia (5% CO_2, 1% O_2) for three days before being harvested for further analysis. In parallel experiments, we assessed the efficiency of PAC transfection by transfecting fluorescently-labeled miR-mimic (miR-mimic-Pe-Cy3) (Applied Biosystems). The percentage of transfected PACs was greater than 95%.

Human umbilical vein endothelial cells (HUVECs) were purchased by Invitrogen and cultured in EBM-2 medium, supplemented with EGM-2 SingleQuots (Lonza) and 2% FBS (GIBCO).

RNA extraction and expressional analyses

RNA was extracted from cultured cells using miRNeasy Mini Kit (Qiagen) following the manufacturer's instructions. The concentration of total RNA was determined using the Nanodrop ND1000 Spectrophotometer (Thermo Scientific). RNA reverse transcription to measure miRs was performed with the TaqManmiR reverse transcription kit following the manufacturer's instructions (Applied Biosystems). MiR-210 (hsa-mir-210, assay ID: 000512) expression was analyzed by the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) and normalized to the U6 small nucleolar RNA (U6 snRNA, assay ID:001973). For gene expression analyses, single-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using TaqMan Reverse Transcription reagents (Applied Biosystems). Quantitative RT-PCR was performed with the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) using the following primers: 18s rRNA (forward: 5-CGCAGCTAGGAATAATGGAATAGG-3'; reverse: 5'-CATGGCCTCAGTTCCGAAA-3'), CXCR4 (forward: 5'-CAGTGGCCGACCTCCTCTT-3'; reverse: 5'- GGACTGCCTTGCATAGGAAGTT-3'); EFNA3 (forward: 5'-CTCTCCCCCAGTTCACCAT-3'; reverse: 5'-TGAGGGTTCTCTCCCTCAAA-3') VEGF-A (forward: 5'-CAACATCACCATGCAGATTATGC-3'; reverse: 5'-TCGGCTTGGCACATTTTTCTTGT-3'); ALDO (forward: 5'-GGCTGCCACTGAGGAGTTC3' reverse: 5'-CTGCTGCTCCACCATCTTCT-3') GLUT-1 (forward: 5'-GATTGGCTCCTTCTCTGTGG-3'; reverse: 5'-TCAAAGGACTTGCCCAGTTT-3') ADM (forward: 5'-GGAAGAGGGAACTGCGGATGT-3'; reverse: 5'-GGCATCCGGATTCACCCTCCTAGC-3'). Data were normalized to 18S ribosomal RNA as an endogenous control. For miR-210 and gene expression, each PCR reaction was performed in duplicate and analyzed by the 2−ddCt method.[66, 67] For the investigation of coding and non-coding EFNA3 isoforms and EPHA4the following primers pair were designed: coding EFNA3 (forward: 5'-CACTCTCCCCCAGTTCACCAT -3'; reverse: 5'-CGCTGATGCTCTTCTCAAGCT-3') non-coding EFNA3 (forward: 5'-AGTTTGGGCTGCGGAGAATC-3'; reverse: 5'-GCAGACGAACACCTTCATCCT-3'); EPHA4 (forward: 5'-AGCAGCCACTCAGGCAAC-3'; reverse:

5’-ACGAAAATAGGGCGAAATAGAA-3’). Expression was normalized to Ubiquitin C (UCB) housekeeping gene.

FACS analyses

For CXCR4 analysis, PACs ($2x10^5$ cells) were stained with 5 µL of CXCR4 (APC) antibody (BD Biosciences). After 15 min incubation at room temperature in the dark, cells were washed, resuspended in PBS and analyzed. Cells were analyzed using a FACSCanto flow cytometer with the FACSDiva software (both from BD Biosciences).

For the characterization of peripheral and cord blood PACs, 10^4 cells were incubated with human receptor FC block (eBioscience; 14-9161-71) for 5 min at room temperature. Cells were stained using 1:20 dilution of CD45-APC-Cy7 (BD Biosciences, #560178, 1:20), CD34-PE-Cy7 (BD Biosciences, #348811), KDR-PE (R&D System, FAB357P) and CD45/CD14-FITC/PE (BD Biosciences, BD Simultest 342408) in staining buffer (PBS supplemented with 0.5% bovine serum albumin) for 15 min at room temperature. Cells were washed twice and resuspended in staining buffer before being analysed on AceaNovocyte flow cytometer. Fluorescence minus one control were used to determine positive staining. The quantification of the antigenic profile was performed using the FlowJo v10 software (FlowJo, LLC).

Migration assay

For PACs migration assay 5 µm pore-size filter-equipped transwell chambers (Corning) coated with fibronectin 2 µg/mL were used. Cells ($7.5x10^4$) were placed in the upper chamber and allowed to migrate toward SDF-1α (R&D) (100 ng/mL) or vehicle (control) for 16 hours at 37°C. The cells on the upper part of the filter were scraped away before fixing the filter. The lower side of the filter (containing the migrated cells) was mounted with Vectashield containing DAPI. For each chamber, migrated cells were counted in 5 random fields at 20X magnification. Migration data are expressed as the number of cells migrated per field toward the specific chemoattractant vs. the number of cells migrated both in absence of stimulus and normoxia. Each experiment was performed in

duplicate. Fluorescence was visualized and captured using AXIO OBSERVER A1 microscope equipped with digital image processing software (AxioVision Imaging System), both from Zeiss.

Adhesion assay

HUVECs ($2.5x10^4$) were seeded and cultured for 24 hours in 96-well tissue culture plates (BD Falcon) in complete EGM2 (Lonza) with 2% FBS (GIBCO). PACs ($2.5x10^4$) were PKH26 red-stained (Sigma, stained according to the manufacturer's instructions) and added to the HUVECs layer. After one hour of co-culture at 37°C cells were washed gently with PBS and cell adhesion was quantified by counting adherent red fluorescent-PACs (magnification 10X). Each experiment was performed in duplicate. Fluorescence was visualized and captured using AXIO OBSERVER A1 microscope equipped with digital image processing software (AxioVision Imaging System), both from Zeiss.

In vitro angiogenesis

Capillary-like network formation Assay: $5x10^4$ PACs were added to 8-well chamber slides (Nunc) pre-coated with 150 µL Matrigel (Becton Dickinson), together with $5x10^4$ HUVECs in a total volume of 150 µL EBM- 2 (Lonza) with 0.1% bovine serum albumin (BSA) (Sigma). After 5 hours incubation at 37°C, PACs effect on network formation from HUVECs was measured by counting the number of intersection points in 10 microphotographs of random view fields (magnification 20X). Similar assays were performed adding 50 µL cell conditioned medium (CCM) or 50 µL of PBS containing purified extracellular microvesicles obtained from PACs to the HUVECs in 50 µL EBM-2 0.1% BSA. For double stained pictures $5x10^4$ HUVECs and $5x10^4$ PACs were stained with PKH26 red and PKH67 green respectively according to the manufacturer's advices prior to co-culture on matrigel. The assays were performed in duplicate wells. Microphotographs were visualized and captured using AXIO OBSERVER A1 microscope equipped with digital image processing software (AxioVision Imaging System), both from Zeiss.

Extracellular microvesicles (EVs) isolation and quantification

Cell conditioned medium (CCM) was processed for EV collection and ultrapurification, as described.[68] For CCM, cells were removed by centrifugation (500g, 5 min), then CCM was clarified by centrifugation (2000g, 30 min followed by 12000g, 45 min at 4°C). EVs were collected by ultracentrifugation (110000g, 3 hours), washed in PBS and pelleted. The purified EV fraction was re-suspended in 100 µL PBS for use. EV purity and amount (normalized on the number of cultured cells) were confirmed by NanoSight technology.[69]

Immunofluorescence analysis

PACs were washed with PBS and fixed with 4% paraformaldehyde (Electron Microscopy Sciences). Cells were then incubated with 10% goat serum (Sigma) at 25˚C for 30 minutes, washed with PBS and incubated with a rabbit anti-EFNA3 antibody (1:300) in PBS overnight at 4˚C. PACs were then washed with PBS and incubated with Goat anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor 555 conjugate (TermoFisher Scientific) (1:200) in PBS for 1 hour at 37˚C. For phalloidin staining cells were permeabilized with 0.1% Triton X-100, following fixing, then washed, blocked with 10% goat serum as described above and incubated 40 minutes with Tetramethylrhodamine B isothiocyanate conjugated phalloidin which directly binds cytoskekletal F-actin. Fluorescence was visualized and captured using AXIO OBSERVER A1 microscope equipped with digital image processing software (AxioVision Imaging System), both from Zeiss.

Lentiviral packaging, preparation and transduction of PACs

Human embryonic kidney cells (HEK293T) were purchased by Sigma and cultured accordingly to Sigma protocols in DMEM 4.5 g/L glucose and 10% FBS (GIBCO) with sodium pyruvate 100 mM (100x), NEAA (100x), penicillin streptomycin (100x) (all from Sigma). The day before, $2.5x10^6$ HEK293T were plated in 100mm dishes (BD Flacon); when cells reached 70-80% confluence, fresh medium was replaced. For transfection two tubes were prepared each with CaCl2 2.5 M, pREV (0.64 µg), pMDL (0.64 µg), pVSVG (0.64 µg) and pEFNA3 (3.2 µg) or pCTRL (3.2 µg). Each

tube was then mixed with HBS 2X and incubated for 15 minutes at 25˚C. Each mixture was used for transfection of HEK293T. Lentivirus titration was performed by transducing HEK293T cells with concentrated particles in the presence of 4 µg/ml polybrene and measuring GFP expression after 3 days by flow cytometry. Cells were then incubated overnight at 37˚C, then the medium was replaced and 24 hours later cells supernatants containing the virus were harvested and filtered using a 0.45 µm sterile filters (Millipore).

PACs were transduced with lentivirus at 1 MOI concentration 24 hours after the end of the transfection with miR-210. The day of transduction PACs were washed with PBS and EFNA3 (or CTRL) lentivirus plus polybrene (final concentration 8 µg/mL) (Sigma) were added. 4 hours after infection, an equal volume of complete PACs medium was added. Cells were selected with puromycine (1 µg/mL) (Calbiochem) after 48 hours from transduction.

Surgical model of limb ischemia in mice

The experiments involving mice were performed in accordance with the Animal (Scientific Procedures) Act (UK) of 1986 prepared by the Institute of Laboratory Animal Resources and covered under the UK Home Office Project licence PPL/30/2811 and personal licenses. Experiments were performed by a blind investigator. Three-month-old immunocompromised CD1-Foxn1nu male mice (Charles River, United Kingdom; n=12–14 mice/group) or C57BL6 mice (Charles River, Italy, n=3-5/group) underwent surgical induction of unilateral limb ischemia (LI) by performing dissection of the left femoral artery, as we reported previously.[70] Immediately after LI induction, CD1-Foxn1nu mice received $2x10^5$ PACs from cord blood from 3 different donors, injected into the adductor muscle. The superficial blood flow to both ischemic and non-ischemic feet was measured using a high-resolution laser color Doppler imaging system (Moor LDI2, Moor Instruments) in anesthetised animals with 1% isoflurane at days 0, 7, 14 and 21 after induction of limb ischemia. The ratio of blood flow between the ischemic and contralateral foot was calculated to use as an index of percentage blood flow recovery. At day 21, mice under terminal anaesthesia were perfusion-fixed successively with 6 mL of 0.05 M EDTA and 10 mL of 10% formalin solution.

Limb muscles were harvested and stored in PFA 4% overnight at room temperature then washed with PBS and finally treated with 30% sucrose overnight at 4°C. The tissue samples were then embedded in optical cutting temperature (OCT) compound and stored at −80°C until histological and immunohistochemical analyses. C57BL6 mice were used for plasma miR experiments, as previously reported.[71]

Immunohistology analysis on ischemic limb muscles

The functional impact of engineered PACs on treatment of CD1-Foxn1nu ischemic mice was assessed by measuring capillaries and arterioles density in the adductor muscle. Eight-micrometer-thick muscle sections were stained using biotin-conjugated Isolectin B4 (from GriffoniaSimplicifolia; Thermo Fisher Scientific) and streptavidin-conjugated Alexa 488 (Thermo Fisher Scientific) antibodies to detect capillaries. Arterioles were identified by staining of α-smooth muscle actin (α- SMA-Cye3, Sigma) and Wheat germ agglutinin-Alexa 647 (Thermo Fischer Scientific) was used to count the muscle fibres. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole). The slides were mounted using Fluoromount-G (eBioscience). The relative number of positive cells was counted in ten randomly selected high-power fields (magnification 200×) using a Zeiss inverted fluorescence microscope. Analyses were performed using muscles from eight mice per group. Capillary density was expressed in as capillary number to myofibre number ratio. Arteriole density was expressed as number per mm square. Quantification was conducted by investigator blinded.

Statistical analyses

Data are presented as mean±SEM and N refers to the number of donors of cells for *in vitro* experiments and number of animals for the *in vivo* analyses that are indicated in the figure legends. Statistical analyses and graphical representations were performed with appropriate software (GraphPad Prism). For molecular characterization and functional assessment of PACs, paired t test was used. Significance level was set at $p<0.05$. For statistical comparison in animal

studies, correlated outcome analysis was used to measure differences in between groups in blood flow recovery in the in vivo model of mouse critical limb ischemia. For histology analysis of capillary and arteriole density, one-way ANOVA with post-hoc Dunnett's test was used.

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Author contribution

Conceptualization, F.M., G.S., C.E., Methodology, G.S., F.M., C.E., S.G., E.S.; Investigation, S.G., E.S., R.V., L.C., M.B., M.C., V.B., B.M., G.Z., S.A., P.F., A.F., M.S., D. DS.; Writing – Original Draft, S.G., M.B., R.V., G.S.; Writing – Review and Editing, G.S., C.E., F.M.; P.M., Supervision, F.M., C.E., P.M., G.S.; Funding Acquisition, F.M., C.E., P.M..

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Figure legends

Figure 1. Hypoxia enhances miR-210 expression in culture-selected human PACs from peripheral blood. A) Cytofluorimetric characterization of peripheral blood (PB)-PACs. The bar graph shows the percentage of cells positive for hematopoietic cell marker CD45, pro-angiogenic cell markers CD34 and KDR, and monocyte marker CD14 (N=4 donors). **B**) Bar graph of average relative expression of (**i**) primary (pri)-miR-210 and (**ii**) mature miR-210 in PB-PACs to U6 snRNA which was used as a normalyzer; (**iii**) miR-210 expression vs. cel-39 spike-in gene in the unfractionated cell medium (CCM) of PB-PACs and (**iv**) in its extracellular vesicles (EVs); PACs were cultured 48 hours under normoxia (white bars) or hypoxia (grey bars, at 1%O_2); *p<0.05 and **p<0.01 vs. normoxia, N=4 donors.

Figure 2. miR-210 affects the migratory capacity and morphology of PB-PACs. A) Bar graph shows the capacity of PB-PACs engineered with pre- or anti-miR-210 to migrate toward SDF-1 (CXCL-12) or in the absence of chemoattratant (vehicle). *p<0.05, **p<0.01 ***p<0.001 vs. vehicle; $^{\#}$p<0.05, $^{\#\#}$p<0.01 vs. SCR. N=6 donors. **B**) Alteration of cell shape in response to miR-210 modulation. Representative images of phalloidin staining: round shaped cells are indicated by yellow arrows. Scale bar 50 µm. Bar graph indicates the average number of round shaped cells , *p<0.05. N=5 donors.

Figure 3. miR-210 mediates PB-PACs interaction with endothelial cells. A) miR-210-transfected-PB-PAC adhesion assay to human umbilical vein endothelial cells (HUVECs). Bar graph of average cell number (left) and representative images (right, scale bar 100µm). N=3 donors; **B**) Bar graph of average intersections of HUVECs cultured in the presence of engineered PB-PACs (bottom panel) and representative images (upper panels, scale bar 100µm). Red= PKH26 dye (HUVECs); Green=PKH67 dye (PACs). N=4 donors. *p<0.05; ***p<0.001

Figure 4. Analysis of coding and non-coding EFNA3 isoforms following miR-210 modulation. A) The expression of both coding and non-coding EFNA3 has been analyzed following miR-210 modulation in PB-PACs under normoxia and hypoxia. Real time q-PCR showed no mRNA modulation for coding (**i**) and non-coding EFNA3 over UCB housekeeping gene. N=4donors). **B**) Coding EFNA3 quantification by immunofluorescence staining confirmed EFNA3 is controlled by miR-210 modulation under normoxia but not hypoxia. N=4 donors, ** p<0.01 vs. SCR normoxia and pre-miR-210 normoxia.

Figure 5. mir-210 over-expression mediates PB-PACs migratory and adhesive functions which are impaired by concomitant EFNA3 over-expression. A) Analysis of miR-210 expression in PACs infected for 24h with EFNA3 bearing lentivirus (LV) and trasfected with pre-miR-210. Bars represent treatment respectively with empty vector lentivirus (white bar), empty vector lentivirus + pre-miR-210 (grey bar), EFNA3 lentivirus (orange bar) and EFNA3 lentivirus + pre-miR-210 (red bar). Data expressed as mean 2ddCts±SEM vs. U6 snRNA. Concentration of viral particles 1 MOI. N=5 donors. *p<0.05, **p<0.01**. B**) Analysis of EFNA3 mRNA by qPCR in PACs treated as described in A). Data expressed as mean 2ddCts±SEM vs. U6 snRNA. N=4 donors. *p<0.05. Lentivirus-mediated EFNA3 overexpression in PB-PACs abrogated the positive impacts induced by miR-210 overexpression on migration (**C**) and adhesion (**D**)**.** For panel C) *p<0.05 vs. vehicle, #p<0.05 vs. SCR. N=4 donors. For panel D) *p<0.05, **p<0.01, ***p<0.001; N=5 donors

Figure 6. UBC-PACs engineered ex vivo to overexpress miR-210 show an higher therapeutic potential for vascular repair in an immunocompromised mouse model of unilateral limb ischemia. UBC-PACs were engineered ex vivo with scramble control (SCR), pre- or anti miR-210. Engineered UBC-PACs or fresh cell medium were injected in the ischemic adductor muscle of mice with surgically induced unilateral limb ischemia.**A**) Postischemic blood flow recovery: (**i**) Time course of blood flow recovery (calculated as the ratio of blood flow in ischemic to contralateral foot)

(N=12–14/group), (**ii**) representative color laser Doppler flowmetry images at 0 and 21 days from surgery. **B**)Analyses of capillary and arteriole density in ischemic muscles: (i) bar graphs showing capillary and arteriole (≤20 μm diameter) density in ischemic adductor muscles at 21 days postischemia (N=8-10/group). (**ii**) Representative ischemic muscle sections stained with the endothelial marker isolectin B4 (green fluorescent) and an antibody for α-smooth muscle actin (red fluorescent) to identify smooth muscles cells in the arterial wall (scale bar, 100 μm). Arterioles are shown by white arrows. Data are expressed as mean±SEM. * $p<0.05$, **$p<0.01$, PACs + pre-miR-210 vs. medium; † †, $p<0.01$, PACs + pre-miR-210 vs. PACs + SCR; #, $p<0.05$, PACs + pre-miR-210 vs. PACs + anti-miR-210**.**